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(54) Title: METHOD FOR DETECTION OF TARGET NUCLEIC ACIDS USING PCR

(57) Abstract

A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising (a) subjecting said sample to an amplification reaction using a set of nucleotides, at least one of which is fluorescently labelled, (b) contacting amplification product with a probe under conditions in which the probe will hybridise to said target sequence, said probe comprising a reactive molecule which is able to absorb fluorescence from or donate fluorescent energy to said fluorescent labelled nucleotide and (c) monitoring fluorescence of said sample. The method can be used to quantitate the amount of target nucleic acid in the sample as well as to determine sequence characteristics. Kits for effecting the method are also claimed.

METHOD FOR DETECTION OF TARGET NUCLEIC ACIDS USING PCR

The present invention provides a method for detecting a target
polynucleotide in a sample, for example by monitoring an
5   amplification reaction, preferably in a quantitative manner,
as well as to probes and kits for use in these methods. The
method is also suitable for the detection of sequence
characteristics such as polymorphisms or allelic variation and
so may be used in diagnostic methods
10

Known fluorescence polymerase chain reaction (PCR) monitoring
techniques include both strand specific and generic DNA
intercalator techniques that can be used on a few second-
generation PCR thermal cycling devices.
15

Generic methods utilise DNA intercalating dyes that exhibit
increased fluorescence when bound to double stranded DNA
species. Fluorescence increase due to a rise in the bulk
concentration of DNA during amplifications can be used to
20   measure reaction progress and to determine the target molecule
copy number. Furthermore, by monitoring fluorescence with a
controlled change of temperature, DNA melting curves can be
generated, for example, at the end of PCR thermal cycling.

25   Generic DNA methods monitor the rise in bulk concentration of
nucleic acids without any time penalty. A single fluorescent
reading can be taken at the same point in every reaction.
End point melting curve analysis can be used to discriminate
artefacts from amplicon, and to discriminate amplicons.
30   Peaks of products can be seen at concentrations that cannot be
visualised by agarose gel electrophoresis.

In order to obtain high resolution melting data, the melt
experiment must be performed slowly on existing hardware
35   taking up to five minutes. However, by continually monitoring
fluorescence amplification, a 3D image of the hysteresis of
melting and hybridisation can be produced. This 3D image is

amplicon dependent and may provide enough information for
product discrimination.

It has been found that DNA melting curve analysis in general
5   is a powerful tool in optimising PCR thermal cycling. By
determining the melting temperatures of the amplicons, it is
possible to lower the denaturing temperatures in later PCR
cycles to this temperature. Optimisation for amplification
from first generation reaction products rather than the
10   genomic DNA, reduces artefact formation occuring in later
cycles. Melting temperatures of primer oligonucleotides and
their complements can be used to determine their annealing
temperatures, reducing the need for empirical optimisation.

15   The generic intercalator methods however are only quasi-
strand-specific and are therefore not very useful where strand
specific detection is required.

Strand specific methods utilise additional nucleic acid
20   reaction components to monitor the progress of amplification
reactions. These methods may use fluorescence energy transfer
(FET) as the basis of detection. One or more nucleic acid
probes are labelled with fluorescent molecules, one of which
is able to act as an energy donor and the other of which is an
25   energy acceptor molecule. These are sometimes known as a
reporter molecule and a quencher molecule respectively. The
donor molecule is excited with a specific wavelength of light
for which it will normally exhibit a fluorescence emission
wavelength. The acceptor molecule is also excited at this
30   wavelength such that it can accept the emission energy of the
donor molecule by a variety of distance-dependent energy
transfer mechanisms. A specific example of fluorescence
energy transfer which can occur is Fluorescence Resonance
Energy Transfer or "FRET". Generally the acceptor molecule
35   accepts the emission energy of the donor molecule when they
are in close proximity (e.g. on the same, or a neighbouring
molecule). The basis of FET or FRET detection is to monitor
the changes at donor and acceptor emission wavelengths.

There are two commonly used types of FET or FRET probes, those
using hydrolysis of nucleic acid probes to separate donor from
acceptor, and those using hybridisation to alter the spatial
relationship of donor and acceptor molecules.

Hydrolysis probes are commercially available as TaqMan™
probes. These consist of DNA oligonucleotides which are
labelled with donor and acceptor molecules. The probes are
designed to bind to a specific region on one strand of a PCR
product. Following annealing of the PCR primer to this
strand, *Taq* enzyme extends the DNA with 5' to 3' polymerase
activity. *Taq* enzyme also exhibites 5' to 3' exonuclease
activity. TaqMan™ probes are protected at the 3' end by
phosphorylation to prevent them from priming *Taq* extension.
If the TaqMan™ probe is hybridised to the product strand than
an extending *Taq* molecule may also hydrolyse the probe,
liberating the donor from acceptor as the basis of detection.
The signal in this instance is cumulative, the concentration
of free donor and acceptor molecules increasing with each
cycle of the amplification reaction.

The fact that signal generation is dependent upon the
occurrence of probe hydrolysis reactions means that there is a
time penalty associated with this method. Furthermore, the
presence of the probe may interrupt the smooth operation of
the PCR process.

In addition, it has been found that hydrolysis can become non-
specific, particularly where large numbers of amplification
cycles, for instance more than 50 cycles, are required. In
these cases, non-specific hydrolysis of the probe will result
in an unduly elevated signal.

This means that such techniques are not very compatible with
rapid PCR methods which are becoming more prominent with the
development of rapid hot air thermal cyclers such as the
RapidCycler™ and LightCycler™ from Idaho Technologies Inc.

Other rapid PCR devices are described for example in co-
pending British Patent Application Nos. 9625442.0 and
9716052.7. The merits of rapid cycling over conventional
thermal cycling have been reported elsewhere. Such techniques
5   are particularly useful for example in detection systems for
biological warfare where speed of result is important if loss
of life or serious injury is to be avoided.

Furthermore, hydrolysis probes do not provide significant
10   information with regard to hysteresis of melting since signal
generation is, by and large, dependent upon hydrolysis of the
probe rather than the melt temperature of the amplicon.

Hybridisation probes are available in a number of guises.
15   Molecular beacons are oligonucleotides that have complementary
5' and 3' sequences such that they form hairpin loops.
Terminal fluorescent labels are in close proximity for FRET to
occur when the hairpin structure is formed. Following
hybridisation of molecular beacons to a complementary sequence
20   the fluorescent labels are separated, so FRET does not occur,
and this forms the basis of detection.

Pairs of labelled oligonucleotides may also be used. These
hybridise in close proximity on a PCR product strand bringing
25   donor and acceptor molecules together so that FRET can occur.
Enhanced FRET is the basis of detection. Variants of this
type include using a labelled amplification primer with a
single adjacent probe.

30   The use of two probes, or a molecular beacon type of probe
which includes two labelling molecules increases the cost
involved in the process. In addition, this method requires
the presence of a reasonably long known sequence so that two
probes which are long enough to bind specifically in close
35   proximity to each other are known. This can be a problem in
some diagnostic applications, where the length of conserved
sequences in an organism which can be used to design an

effective probe, such as the HIV virus, may be relatively
short.

Furthermore, the use of pairs of probes involves more complex
5   experimental design. For example, a signal provided when by
the melt of a probe is a function of the melting off of both
probes. The study of small mismatches or where one of the
probes is required to bind across a splice region (for example
to detect RNA as compared to DNA in a sample where the
10   sequence on either side of an intron can be utilised as the
probe site) can yield incorrect results if the other probe
melts first.

The applicants have developed an assay for detecting the
15   presence of particular nucleic acid sequences which may be
adapted to quantify the amount of the target sequence in the
sample.

Thus the invention provides a method for detecting the
20   presence of a target nucleic acid sequence in a sample, said
method comprising (a) subjecting said sample to an
amplification reaction using a set of nucleotides, at least
one of which is fluorescently labelled, (b) contacting
amplification product with a probe under conditions in which
25   the probe will hybridise to said target sequence, said probe
comprising a reactive molecule which is able to absorb
fluorescence from or donate fluorescent energy to said
fluorescent labelled nucleotide and (c)monitoring fluorescence
of said sample.
30
Using an assay of this type, a fluorescent label becomes
incorporated only into amplification product. When the probe
hybridises to any specific target sequence produced as a
result of the amplification reaction, the reactive molecule
35   absorbs emission energy from labelled nucleotides or donates
energy to the labelled nucleotides by means of FET or FRET,
thus changing the signal from the fluorescent nucleotides.
Suitably, the reactive molecule is able to absorb fluorescence

from the labelled nucleotides and so the fluorescence from
these is reduced. This reduction may be detected and this
indicates binding of the probe.

5   Most preferably, the reactive molecule is an acceptor molecule
     which it emits fluorescence at a characteristic wavelength.
     In this case, increase in fluorescence from the acceptor
     molecule, which is of a different wavelength to that of the
     labelled nucleotide, will also indicate binding of the probe.

10

     The presence of the thus labelled amplification product can be
     detected by monitoring fluorescence from the acceptor molecule
     on the probe, which specifically binds only the target
     sequence. In this case, signal from the amplification product

15   can be distinguished from background signal of the fluorescent
     label and also from any non-specific amplification product.

     The fact that the signal is partly associated with the
     amplification product and partly associated with the probe

20   means that the system is highly specific in terms of detecting
     specific target sequences in reaction mixtures that contain
     large amounts of background DNA. This is because signal from
     non-specific amplification product can effectively be
     eliminated from the measured signal.

25

     An assay of this nature can be carried out using inexpensive
     reagents. Single labelled probes are more economical to those
     which include both acceptor and donor molecules.

30   As used herein, the expression "set of nucleotides" refers to
     a group of nucleotides which are sufficient to form nucleic
     acids such as DNA and RNA. Thus these comprise adenosine,
     cytosine, guanine and thymine or uracil. One or more of these
     is fluorescently labelled. Labelled uracil is available from

35   Boehringer Mannheim. Suitable fluorescent labels include
     fluorescein.

The use of labelled uracil may be particularly preferred in that its use may be built into the a strategy for preventing contamination or carry-over from one amplification reaction to subsequent ones carried out in the reaction vessels. Enzymes

5   which digest uracil containing nucleic acids, such as uracil-N-glycosylase, can be used in a pre-cycling incubation step, to ensure that any residual amplicons are digested before thermal cycling in the subsequent application begins.

10 Suitably more than one nucleotide, and most preferably all the nucleotides are labelled as this will moderate the level of signal from the amplification product and thus the FET or FRET signal.

15 Amplification is suitably effected using known amplification reactions such as the polymerase chain reaction (PCR) or the ligase chain reaction (LCR), strand displacement assay (SDA) or NASBA, but preferably PCR.

20 Preferably, the fluorescence of both the nucleotide and the acceptor molecule are monitored and the relationship between the emissions calculated.

    Suitable reactive molecules (such as acceptor molecules) are
25 rhodamine dyes or other dyes such as Cy5. These may be attached to the probe in a conventional manner. The position of the reactive molecule along the probe is immaterial although it general, they will be positioned at an end region of the probe.
30
    In order for FET, such as FRET, to occur between the reactive molecule and fluorescent emission of the nucleotides, the fluorescent emission of the element (reactive molecule or labelled nucleotide) which acts as the donor must be of a
35 shorter wavelength than the element acceptor.

    Suitable combinations are therefore set out in the following Table:

Donor	Acceptor
SYBRGold	rhodamine
SYBRGreen I	rhodamine
SYBRGold	Cy5
SYBRGreen I	Cy5
Fluorescein	Ethidium bromide

Preferably, the molecules used as donor and/or acceptor
5   produce sharp peaks, and there is little or no overlap in the
wavelengths of the emission. Under these circumstances, it
may not be necessary to resolve the "strand specific peak"
from the signal produced by amplification product. A simple
measurement of the strand specific signal alone (i.e. that
10   provided by the reactive molecule) will provide information
regarding the extent of the FET or FRET caused by the target
reaction. The ethidium bromide/fluorescein combination may
fulfil this requirement. In that case, the strand specific
reaction will be quantifiable by the reduction in fluorescence
15   at 520nm, suitably expressed as 1/Fluorescence.

However, where there is a spectral overlap in the fluorescent
signals from the donor and acceptor molecules, this can be
accounted for in the results, for example by determining
20   empirically the relationship between the spectra and using this
relationship to normalise the signals from the two signals.

The method of the invention is extremely versatile in its
applications. The method can be used to generate both
25   quantitative and qualitative data regarding the target nucleic
acid sequence in the sample, as discussed in more detail
hereinafter. In particular, not only does the invention
provide for quantitative amplification, but also it can be
used, additionally or alternatively, to obtain characterising
30   data such as duplex destabilisation temperatures or melting
points.

In the method of the invention, the sample may be subjected to
conditions under which the probe hybridises to the samples
during or after the amplification reaction has been completed.
5   The process allows the detection to be effected in a
homogenous manner, in that the amplification and monitoring
can be carried out in a single container with all reagents
added intially. No subsequent reagent addition steps are
required. Neither is there any need to effect the method in
10 the presence of solid supports (although this is an option as
discussed further hereinafter).

For example, where the probe is present throughout the
amplification reaction, the fluorescent signal may allow the
15 progress of the amplification reaction to be monitored.
This may provide a means for quantitating the amount of target
sequence present in the sample.

During each cycle of the amplification reaction, amplicon
20 strands containing the target sequence bind to probe and
thereby generate an acceptor signal. As the amount of
amplicon in the sample increases, so the acceptor signal will
increase. By plotting the rate of increase over cycles, the
start point of the increase can be determined.
25

The probe may comprise a nucleic acid molecule such as DNA or
RNA, which will hybridise to the target nucleic acid sequence
when the latter is in single stranded form. In this instance,
step (b) will involve the use of conditions which render the
30 target nucleic acid single stranded. Alternatively, the probe
may comprise a molecule such as a peptide nucleic acid which
specifically binds the target sequence in double stranded
form.

35 In particular, the amplification reaction used will involve a
step of subjecting the sample to conditions under which any of
the target nucleic acid sequence present in the sample becomes
single stranded, such as PCR or LCR.

It is possible then for the probe to hybridise during the
course of the amplification reaction provided appropriate
hybridisation conditions are encountered.

5   In a preferred embodiment, the probe may be designed such that
these conditions are met during each cycle of the
amplification reaction. Thus at some point during each cycle
of the amplification reaction, the probe will hybridise to the
target sequence, and generate a signal as a result of the FET
10   or FRET. As the amplification proceeds, the probe will be
separated or melted from the target sequence and so the signal
generated by the reactive molecule will either reduce or
increase depending upon whether it comprises the donor or
acceptor molecule. For instance, where it is an acceptor, in
15   each cycle of the amplification, a fluorescence peak from the
reactive molecule is generated. The intensity of the peak
will increase as the amplification proceeds because more
target sequence becomes available for binding to the probe.

20   By monitoring the fluorescence of the reactive molecule from
the sample during each cycle, the progress of the
amplification reaction can be monitored in various ways. For
example, the data provided by melting peaks can be analysed,
for example by calculating the area under the melting peaks
25   and this data plotted against the number of cycles.

Fluorescence is suitably monitored using a known fluorimeter.
The signals from these, for instance in the form of photo-
multiplier voltages, are sent to a data processor board and
30   converted into a spectrum associated with each sample tube.
Multiple tubes, for example 96 tubes, can be assessed at the
same time. Data may be collected in this way at frequent
intervals, for example once every 10ms, throughout the
reaction.
35

The spectra generated in this way can be resolved, for
example, using "fits" of pre-selected fluorescent moieties
such as dyes, to form peaks representative of each signalling

moiety (i.e. nucleotide label and/or reactive molecule). The
areas under the peaks can be determined which represents the
intensity value for each signal, and if required, expressed as
quotients of each other. The differential of signal

5   intensities and/or ratios will allow changes in FET or FRET to
be recorded through the reaction or at different reaction
conditions, such as temperatures. The changes, as outlined
above, are related to the binding phenomenon between the probe
and the target sequence. The integral of the area under the

10   differential peaks will allow intensity values for the FET or
FRET effects to be calculated.

This data provides one means to quantitate the amount of
target nucleic acid present in the sample.

15
The probe may either be free in solution or immobilised on a
solid support, for example to the surface of a bead such as a
magnetic bead, useful in separating products, or the surface
of a detector device, such as the waveguide of a surface

20   plasmon resonance detector. The selection will depend upon
the nature of the particular assay being looked at and the
particular detection means being employed.

The probe may be designed such that it is hydrolysed by the

25   DNA polymerase used in the amplification reaction thereby
releasing the acceptor molecule. This provides a cumulative
signal, with the amount of free reactive molecule present in
the system increasing with each cycle. However, it is not
necessary in this assay for the probe to be consumed in this

30   way as the signal does not depend upon the hydrolysis of the
probe.

In order to achieve a fully reversible signal which is
directly related to the amount of amplification product

35   present at each stage of the reaction, and/or where speed of
reaction is of the greatest importance, for example in rapid
PCR, it is preferable that the probe is designed such that it
is released intact from the target sequence and so may take

part again in the reaction. This may be, for example, during
the extension phase of the amplification reaction. However,
since the signal is not dependent upon probe hydrolysis, the
probe may be designed to hybridise and melt from the target
5   sequence at any stage during the amplification cycle,
including the annealing or melt phase of the reaction. Such
probes will ensure that interference with the amplification
reaction is minimised.

10 Where probes which bind during the extension phase are used,
their release intact may be achieved by using a 5'-3'
exonuclease lacking enzyme such as Stoffle fragment of Taq or
Pwo. This may be useful when rapid PCR is required as
hydrolysis steps are avoided.
15

When used in this way, it is important to ensure that the
probe is not extended during the extension phase of the
reaction. Therefore, the 3' end of the probe is blocked,
suitably by phosphorylation.
20

The data generated in this way can be interpreted in various
ways. In its simplest form, an increase in fluorescence of
the acceptor molecule in the course of or at the end of the
amplification reaction is indicative of an increase in the
25 amount of the target sequence present, suggestive of the fact
that the amplification reaction has proceeded and therefore
the target sequence was in fact present in the sample.
However, as outlined above, quantitation is also possible by
monitoring the amplification reaction throughout. Finally, it
30 is possible to obtain characterisation data and in particular
melting point analysis, either as an end point measure or
throughout, in order to obtain information about the sequence
as will be discussed further below.

35 Thus, a preferred embodiment of the invention comprises a
method for detecting nucleic acid amplification comprising:
performing nucleic acid amplification on a target
polynucleotide in the presence of (a) a nucleic acid

polymerase (b) at least one primer capable of hybridising to
said target polynucleotide, (c) a set of nucleotides, at least
one of which is fluorescently labelled and (d) an
oligonucleotide probe which is capable of binding to said
5   target polynucleotide sequence and which contains a reactive
molecule which is capable of absorbing fluorescence from or
donating fluorescence to the said labelled nucleotide; and
monitoring changes in fluorescence during the amplification
reaction.   Suitably, the reactive molecule is an acceptor
10   molecule which can absorb energy from the labelled nucleotide.

The amplification is suitably carried out using a pair of
primers which are designed such that only the target
nucleotide sequence within a DNA strand is amplified as is
15   well understood in the art.   The nucleic acid polymerase is
suitably a thermostable polymerase such as Taq polymerase.

Suitable conditions under which the amplification reaction can
be carried out are well known in the art.   The optimum
20   conditions may be variable in each case depending upon the
particular amplicon involved, the nature of the primers used
and the enzymes employed.   The optimum conditions may be
determined in each case by the skilled person.   Typical
denaturation temperatures are of the order of 95°C, typical
25   annealing temperatures are of the order of 55°C and extension
temperatures are of the order of 72°C.

In a particular embodiment of the invention the probe may be
used to quantitate RNA transcripts, for example in expression
30   experiments, that maybe used in drug discovery. In particular
this embodiment is suitable for expression studies in tissues
from eukaryotic organisms. DNA encoding proteins in eukaryotic
cells may contain introns, non-coding regions of DNA sequence,
and exons that encode for protein sequence. Non-coding intron
35   sequences are removed from RNA sequences that are derived from
the DNA sequences during cellular "splicing" processes.   PCR
primers are normally targeted at coding regions and when
reverse transcriptase PCR is used on total nucleic acid

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extracts, products will result from both DNA dependent
amplification and RNA dependent amplification. Thus PCR alone,
when used for expression studies, will contain amplification
resulting from genomic DNA and expressed RNA.

5

A probe that is designed to bind across introns, on adjacent
terminal regions of coding exons, will have limited
interaction because of the intron region. Spliced RNA has
these regions removed and therefore the adjacent terminal
10   regions of coding exons form one continous sequence allowing
efficient binding of the probe.

Conversely, a probe may detect only an amplification product
of genomic DNA if it is designed such that it binds an intron
15   region. Signal generated from such a probe would relate only
to the DNA concentration and not the RNA concentration of the
sample.

Thus in a further embodiment, the probe is specific either for
20   a splice region of RNA or an intron in DNA, so that only one
of amplified RNA or amplified DNA is detected and/or
quantitated.

Alternatively or additionally, the method of the invention can
25   be used in hybridisation assays for determining
characteristics of a sequence. Thus in a further aspect, the
invention provides a method for determining a characteristic
of a sequence, said method comprising (a) amplifying said
sequence using a set of nucleotides, at least one of which is
30   fluorescently labelled, (b) contacting amplification product
with a probe under conditions in which the probe will
hybridise to said target sequence, said probe comprising a
reactive molecule which is able to absorb fluorescence from or
donate fluorescent energy to said fluorescent labelled
35   nucleotide and (c)monitoring fluorescence of said sample and
determining a particular reaction condition, characteristic of
said sequence, at which fluorescence changes as a result of
the hybridisation of the probe to the sample or

destabilisation of the duplex formed between the probe and the
target nucleic acid sequence.

Suitable reaction conditions include temperature,
5   electrochemical, or the response to the presence of particular
enzymes or chemicals. By monitoring changes in fluorescence
as these properties are varied, information characteristic of
the precise nature of the sequence can be achieved. For
example, in the case of temperature, the temperature at which
10   the probe separates from the sequences in the sample as a
result of heating can be determined. This can be extremely
useful in for example, to detect and if desired also to
quantitate, polymorphisms and/or allelic variation in genetic
diagnosis. By "polymorphism" is included transitions,
15   transversions, insertions, deletions of inversions which may
occur in sequences, particularly in nature.

The hysteresis of melting will be different if the target
sequence varies by only one base pair. Thus for example,
20   where a sample contains only a single allelic variant, the
temperature of melting of the probe will be a particular value
which will be different from that found in a sample which
contains only another allelic variant. A sample containing
both allelic variants which show two melting points
25   corresponding to each of the allelic variants. Similar
considerations apply with respect to electrochemical
properties, or in the presence of certain enzymes or
chemicals. The probe may be immobilised on a solid surface
across which an electrochemical potential may be applied.
30   Target sequence will bind to or be repulsed from the probe at
particular electrochemical values depending upon the precise
nature of the sequence.

In addition, the kinetics of probe hybridisation will allow
35   the determination, in absolute terms, of the target sequence
concentration. Changes in fluorescence from the sample can
allow the rate of hybridisation of the probe to the sample to
be calculated. An increase in the rate of hybridisation will

relate to the amount of target sequence present in the sample.
As the concentration of the target sequence increases as the
amplification reaction proceeds, hybridisation of the probe
will occur more rapidly. Thus this parameter also can be used
5   as a basis for quantification. This mode of data processing
useful in that it is not reliant on signal intensity to
provide the information.

Further aspects of the invention include kits for use in the
10   method of the invention. These kits will contain a probe
specific for a target nucleotide sequence which contains a
reactive molecule, in particular an acceptor molecule. If
desired, the probe can be immobilised on a support such as a
bead, for example a magnetic bead, or a support used in a
15   detector, such as the waveguide of an evanescent wave detector
device.

Additionally, kits may contain one or more fluorescently
labelled nucleotides which is/are compatible with said
20   reactive molecule. Other potential components of the kit
include reagents used in amplification reactions such as DNA
polymerase.

The invention will now be particularly described by way of
25   example with reference to the accompanying diagrammatic
drawings in which:

Figure 1 shows diagrammatically the interactions which take
place in the method of the invention.
30

In the illustrated amplification reaction, a DNA molecule (1)
prepared for amplification by contacting it with pair of
amplification primers (2), (3) and a set of nucleotides (4)
some of which are labelled with a fluorescent label (5). The
35   DNA molecule (1) is rendered single stranded (Figure 1B)
whereupon the primers (2,3) bind as forward and reverse
primers in an amplification reaction as is well known.

During the course of the subsequent amplification reaction, an
amplicon product (6) is built up (Figure 1C). Nucleotides
both labelled and unlabelled are incorporated into the product
as it is formed. In the normal way, the amplicon product (6)
5   contains only the target sequence as defined by the primers.

When this product is melted during the subsequent phase of the
amplification, probe (7) comprising an acceptor molecule (8)
binds the target sequence (Figure 1D). The FRET interaction
10 between the fluorescent nucleotides and the acceptor molecule
(8) means generates a signal at the wavelength characteristic
of the acceptor.

The acceptor signal (8) can then be monitored using
15 conventional fluorescence detection devices.

Claims

1.   A method for detecting the presence of a target nucleic
acid sequence in a sample, said method comprising (a)
subjecting said sample to an amplification reaction using a
set of nucleotides, at least one of which is fluorescently
labelled, (b) contacting amplification product with a probe
under conditions in which the probe will hybridise to said
target sequence, said probe comprising a reactive molecule
which is able to absorb fluorescence from or donate
fluorescent energy to said fluorescent labelled nucleotide and
(c) monitoring fluorescence of said sample.

2.   A method according to claim 1 wherein in the reactive
molecule absorbs fluorescence from said fluorescent labelled
nucleotide.

3.   A method according to claim 2 wherein the reactive
molecule is an acceptor molecule which emits energy at a
characteristic wavelength.

4.   A method according to claim 3 wherein the acceptor
molecule is a rhodamine dye or other dyes such as Cy5.

5.   A method according to any one of the preceding claims
wherein the labelled nucleotide is labelled uracil.

6.   A method according to any one of the preceding claims
wherein all nucleotides used in the amplification reaction are
labelled.

7.   A method according to any one of the preceding claims
wherein the amplification reaction comprises the polymerase
chain reaction (PCR).

8.   A method according to any one of the preceding claims
wherein the fluorescence of both the labelled nucleotide and

the reactive molecule are monitored and the relationship
between the emissions calculated.

9.   A method according to any one of the preceding claims
5   wherein the probe is present throughout the amplification
reaction.

10.   A method according to claim 9 wherein the fluorescent
signal is monitored and the results used to quantitate the
10   amount of target sequence present in the sample.

11.   A method according to any one of the preceding claims
wherein the probe is designed such that it is hydrolysed by
the DNA polymerase used in the amplification reaction.
15

12.   A method according to any one of claims 1 to 10 wherein
the probe is designed such that during the extension phase of
the amplification reaction, the probe is released intact from
the target sequence.
20

13.   A method according to claim 12 wherein the amplification
reaction is effected using a 5'-3' exonuclease lacking enzyme.

14.   A method according to claim 13 wherein the enzyme is
25   Stoffle fragment of Taq or Pwo.

15.   A method according to any one of claims 12 to 15 wherein
the 3' end of the probe is blocked by phosphorylation.

30   16.   A method for detecting nucleic acid amplification
comprising: performing nucleic acid amplification on a target
polynucleotide in the presence of (a) a nucleic acid
polymerase (b) at least one primer capable of hybridising to
said target polynucleotide, (c) a set of nucleotides, at least
35   one of which is fluorescently labelled and (d)an
oligonucleotide probe which is capable of binding to said
target polynucleotide sequence and which contains a reactive
molecule which is capable of absorbing fluorescence energy

from or donating fluorescent energy to the said labelled
nucleotide; and monitoring changes in fluorescence during the
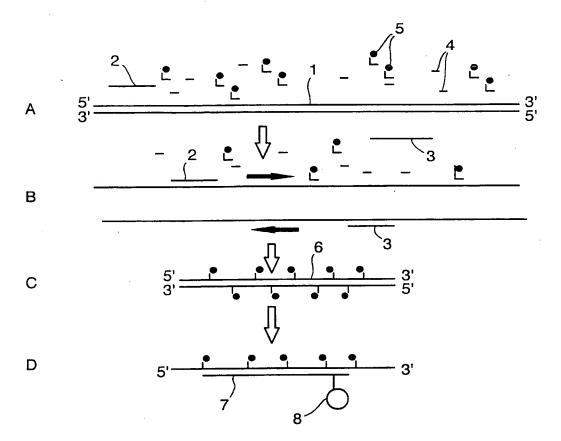amplification reaction.

5   17.   A method according to claim 16 wherein the amplification
is carried out using a pair of primers which are designed such
that only the target nucleotide sequence within a DNA strand
is amplified.

10   18.   A method according to any one of the preceding claims
wherein the probe is specific either for a splice region of
RNA or an intron in DNA, so that only one of amplified RNA or
amplified DNA is detected and/or quantitated.

15   19.   A method for determining a characteristic of a sequence,
said method comprising (a) amplifying said sequence
using a set of nucleotides, at least one of which is
fluorescently labelled, (b) contacting amplification product
with a probe under conditions in which the probe will
20   hybridise to said target sequence, said probe comprising a
reactive molecule which is able to absorb fluorescence from or
donate fluorescent energy to said fluorescent labelled
nucleotide and (c)monitoring fluorescence of said sample
and determining a particular reaction condition,
25   characteristic of said sequence, at which fluorescence changes
as a result of the hybridisation of the probe to the sample or
destabilisation of the duplex formed between the probe and the
target nucleic acid sequence.

30   20.   A method for detecting a polymorphisms and/or allelic
variation, said method comprising amplifying a sequence
suspected of containing said polymorphism or variation using a
method as defined in claim 1, measuring the temperature at
which the probe melts from the amplification product using the
35   fluorescent signal generated, and relating this to the
presence of a polymorphism or allelic variation.

21.   A kit for use in the method of any one of the preceding
claims which comprises a probe specific for a target
nucleotide sequence which contains a reactive molecule and a
fluorescently labelled nucleotide which is compatible with
5   said reactive molecule.

Fig.1.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 46714 A (RASMUSSEN RANDY P ;UNIV UTAH RES FOUND (US); RIRIE KIRK M (US); WI) 11 December 1997 see page 43, paragraph 2 – page 47, paragraph 2 see example 12 see page 60, paragraph 3 – page 66 ---	1-21
A	WO 96 34983 A (PERKIN ELMER CORP) 7 November 1996 see the whole document ---	1-21
A	WO 97 29210 A (PERKIN ELMER CORP) 14 August 1997 see the whole document --- -/--	1-21

| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 May 1999	31/05/1999

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 567 583 A (WANG CHANG-NING J ET AL) 22 October 1996 see the whole document -----	1-21

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

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